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Trafficking of major histocompatibility complex class II molecules in human B-lymphoblasts deficient in the AP-3 adaptor complex

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Abstract

The major histocompatibility complex class II subunits (MHC-II) α and β assemble with the invariant chain (Ii) in the endoplasmic reticulum and are transported to endosomal–lysosomal organelles known as MHC class II compartments (MIICs). Although it has been shown that two dileucine-based signals in the cytosolic tail of Ii, as well as a dileucine-based signal in the tail of the β chain mediate sorting to MIICs, the molecular mechanisms by which αβIi complexes are sorted have yet to be resolved fully. The AP-3 adaptor complex stands out as a particularly good candidate for mediating this targeting because: (i) it has a proven role in the trafficking of membrane proteins to lysosome-related organelles; and (ii) it has the ability to interact with dileucine-based signals in vitro. To investigate the potential role of AP-3 in transport of MHC-II to MIICs, we have examined MHC-II trafficking in human B-lymphoblast lines from patients with Hermansky–Pudlak syndrome type 2 (HPS-2), which are deficient in the AP-3 complex. Pulse-chase analyses revealed no significant alteration in the kinetics of synthesis and degradation of either MHC-II subunits or Ii. Moreover, we observed neither impairment of the formation of compact SDS-resistant αβ dimers, nor delay in the appearance of a conformational epitope indicative of a mature, Ii-free αβ dimer. Finally, we demonstrated that in HPS-2 patients' cells, there was no delay in the expression of the αβ dimers on the cell surface. Thus, AP-3 does not seem to be essential for normal trafficking of MHC-II. These findings have important implications for HPS-2 patients, because they suggest that the recurrent bacterial infections suffered by these patients are not likely due to impaired antigen processing and presentation by MHC-II.

Keywords: Hermansky–Pudlak syndrome; Lysosomes; Antigen-processing; Invariant chain; Dileucine-based signals

1. Introduction

Major histocompatibility complex class II molecules (MHC-II) are heterodimers composed of two transmembrane subunits termed α and β. These subunits are synthesized in the endoplasmic reticulum (ER), where they assemble with a third transmembrane polypeptide referred to as the invariant chain or Ii (reviewed in [1,2]). αβIi chain complexes move from the ER to the Golgi complex and are eventually delivered to compartments of the endosomal–lysosomal system referred to as MHC-II compartments or MIICs [3,4]. Transport to these compartments is mediated by two dileucine-based signals present within the cytosolic domain of Ii [5–7], as well as an additional dileucine-based signal within the cytosolic domain of the β chain [8]. Within MIICs, Ii is removed proteolytically from the αβ dimers leaving a small Ii-fragment known as CLIP attached to the antigen-binding site [9]. Peptide antigens generated by proteolytic degradation of endocytosed antigens subsequently replace CLIP to form a stable complex with the αβ dimer [10]. Peptide-loaded MHC-II molecules then move to the cell surface, where they present the antigenic peptides to T cells.

Although many of the events involved in antigen processing and presentation have been well character-

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ized, the molecular mechanisms by which αβIi complexes are specifically targeted to the endosomal–lysosomal system remain poorly understood. Candidate molecules for this targeting are the heterotetrameric adaptor protein (AP) complexes AP-1, AP-2, AP-3 and AP-4, which play major roles in signal-mediated sorting of integral membrane proteins to compartments of the endosomal–lysosomal system [11]. AP-3, in particular, has been proposed to function in the biogenesis of lysosome-related organelles [12–14], most likely by mediating sorting of integral membrane proteins to those organelles [14,15]. The ubiquitous AP-3 complex is composed of four subunits named δ, β3A, μ3A and σ3 (A or B isoforms). Mutations in the β3A subunit of AP-3 in patients with Hermansky–Pudlak syndrome type 2 (HPS-2) have been shown to cause abnormalities in lysosome-related organelles such as melanosomes and platelet dense granules, as well as enhanced trafficking of lysosomal integral membrane proteins via the cell surface [14]. Similar defects have been observed in the *mocha* and *pearl* mouse strains, which bear mutations in the δ and β3A subunits of AP-3, respectively [12,13]. In addition, AP-3 has been implicated in the targeting of alkaline phosphatase and Vam3p to the yeast vacuole, through recognition of dileucine-based signals present within the cytosolic domains of these proteins [16,17]. Finally, in vitro binding experiments have shown that mammalian AP-3 interacts with the dileucine-based signals of the lysosomal membrane protein LIMP II and the melanosomal protein tyrosinase [18].

Thus, owing to the lysosomal characteristics attributed to MIICs, and the fact that targeting of MHC-II to MIICs depends on dileucine-based signals, there has been widespread speculation that AP-3 may be responsible for sorting of MHC-II to antigen-processing compartments. To test this hypothesis, we have compared the trafficking of MHC-II in B-lymphoblast cell lines from normal individuals and from AP-3-deficient HPS-2 patients.

2. Materials and methods

2.1. Patients and cell culture

Epstein–Barr virus transformed B-lymphoblasts were prepared and propagated as described previously [14]. The donors were normal individuals and HPS-2 patients enrolled in an Institutional Review Board-approved protocol to study Hermansky–Pudlak syndrome [14,19]. Cells were cultured in RPMI1640 medium, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal bovine serum (Biofluids, Rockville, MD).

2.2. Antibodies

Affinity purified anti-β3A -δ, -μ3A and -σ3 antibodies have been described previously [14,20]. The 100/3 monoclonal anti-AP-1-γ antibody was purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal Anti-Ii Pin.1 antibody [10], monoclonal anti-MHC Class II α chain DA6.147 [21], and monoclonal anti-MHC Class II epitope antibody, L243 [22] were used for immunoblotting and immunoprecipitations.

2.3. Immunoblotting

For immunoblot analysis, 5×10^6 cells from either HPS-2 patients or normal individuals were collected and lysed (15 min at 4°C) in a lysis buffer containing 1% (w/v) Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 0.25 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin, 10 mM iodoacetamide. After removal of insoluble matter, whole cell lysates were separated by 4–20% SDS–PAGE, and the proteins were transferred to nitrocellulose filters. After 1h blocking with 5% non-fat milk, the filters were immunoblotted for 2 h with specific antibodies, washed in phosphate-buffered saline (PBS) containing 0.1% Tween 20, and incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min. Bound antibodies were visualized by enhanced chemiluminescence (Amersham).

2.4. Immunoprecipitations and pulse-chase analysis

For pulse-chase studies, 3×10^7 cells were labeled for 20 min at 37°C with 200 μCi/ml [^{35}S]methionine in methionine-free RPMI1640 medium. After three washes, the cells were chased in regular culture medium for the times indicated, washed and lysed as described above. Cell lysates were precleared twice for 1 h, using 15 μl of Protein A-Sepharose. The lysate supernatant was then immunoprecipitated with the designated antibodies and the beads were washed five times in lysis buffer containing 0.1% Triton X-100. Following elution from the beads in 1% SDS, the immunoprecipitates were analyzed by 4–20% SDS–PAGE and fluorography.

For double-label [^{35}S]methionine/biotin pulse-chase experiments, cells were collected after chase at the indicated times, and suspended in 1 ml PBS containing 2 mg/ml D-Biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Roche Molecular Biochemicals) for 2 min at 4°C. After termination of the reaction by the addition of 10 mM Tris, the cells were washed and lysed as described above. To determine levels of cell surface biotinylated proteins, bound proteins were released from Sepharose beads by the addition of 50 μl elution buffer (1% SDS, 1.5 mg/ml dithiothreitol, 10

mM Tris), and incubation for 5 min at room temperature, followed by 5 min at 95°C. Samples were then diluted 20-fold in lysis buffer and subjected to recapture [23] with Streptavidin beads (Pierce). Eluted proteins from the Streptavidin beads were analyzed as above.

3. Results and discussion

Analysis of normal and HPS-2 patients' B-lymphoblasts by immunoblotting revealed that the two patients' cells (W2 and W3) expressed dramatically reduced levels of β3A and μ3A relative to normal cells (WN) (Fig. 1). Levels of δ and σ3 were reduced to a lesser extent (Fig. 1), similar to what had been reported previously for fibroblasts from the same patients [14]. Thus, B-lymphoblasts from HPS-2 patients are unable to assemble a complete AP-3 complex. The levels of γ1-adaptin, one of the subunits of the related AP-1 complex, and of β-actin were similar in the normal and patient cells, confirming the specificity of the AP-3 defects (Fig. 1).

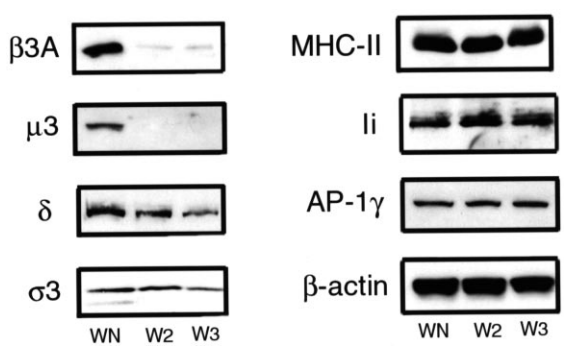

Fig. 1. B-lymphoblasts derived from HPS-2 patients have reduced levels of β3A and μ3A, but not MHC-II or Ii. Immunoblot analysis was performed on whole cell lysates of B-lymphoblasts derived from a normal individual (WN) and two HPS-2 patients (W2 and W3). Samples were normalized using equal cell numbers.

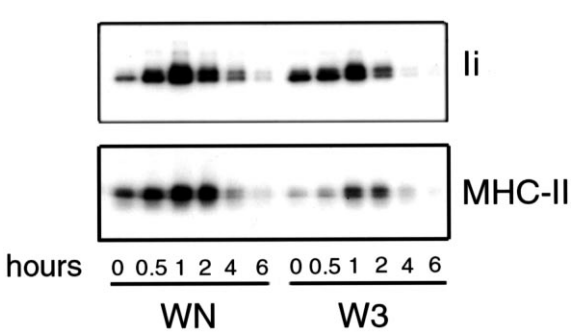

Fig. 2. The kinetics of synthesis and degradation of Ii and MHC-II are similar in cells from HPS-2 patients and normal controls. A total of 30×10^6 cells were pulsed for 20 min with [^{35}S]methionine, and chased for the times indicated. Cell lysates from each time point were then precleared and immunoprecipitations were performed with antibodies directed against Ii or MHC-II.

Immunoblot analysis also showed that the amounts of MHC-II α chain and Ii chain were similar in patient and control cells (Fig. 1), suggesting that AP-3 deficiency did not affect the steady state levels of these proteins. To determine whether the kinetics of transport of αβIi complexes to MIIC were altered, we performed pulse-chase analysis of Ii degradation in normal and patient cells. In both cell lines, detection of Ii increased over the first hour of chase, probably due to the development of an epitope recognized by the antibody used in this experiment (Fig. 2). After the 1st h of chase time, Ii underwent degradation with similar rates in both normal and patient cells. Similar results were obtained for MHC-II chains, utilizing an antibody that recognizes MHC-II α (Fig. 2).

We next examined the formation of SDS-resistant, compact αβ dimers, which reflect loading of MHC-II with peptide antigens in MIICs [24]. The levels of compact dimers observed in non-boiled anti-MHC-II α immunoprecipitates from a pulse-chase experiment were similar in both normal and patient cells (Fig. 3A, arrow). We also determined the appearance of peptide-loaded αβ dimers by immunoprecipitation of samples from a pulse-chase experiment with an antibody (L243) that recognizes Ii-free, peptide-bound dimers [22]. Consistent with the results of the previous experiment, we observed similar rates of appearance of peptide-loaded dimers in normal and patient's cells (Fig. 3B).

Finally, we analyzed the possibility that AP-3 might play a role in transport of peptide-loaded αβ dimers to the cell surface. Normal and patient cells were metabolically-labeled, and then chased for the times indicated in Fig. 4. Following each chase time, the cells were subjected to surface biotinylation. Total mature αβ dimers were immunoprecipitated with L243 antibody, keeping 10% of the sample and reprecipitating the remaining 90% (representing surface mature dimers) with streptavidin beads. The results of this experiment showed no significant difference in the surface/total ratio for patient cells as compared to normal cells (Fig. 4).

Thus, contrary to expectations, the AP-3 deficiency does not appear to significantly impair MHC-II transport to MIICs, or their subsequent delivery to the cell surface. This phenotype of HPS-2 patients differs from that reported for patients suffering from a related disease, Chediak–Higashi syndrome (CHS). Cells from CHS patients do exhibit a delay in MHC-II maturation and peptide loading, as well as in their eventual delivery to the cell surface [25]. Although we cannot completely rule out the possibility that AP-3 plays some role in MHC-II trafficking, this role does not appear to be essential. AP-3 must therefore per-

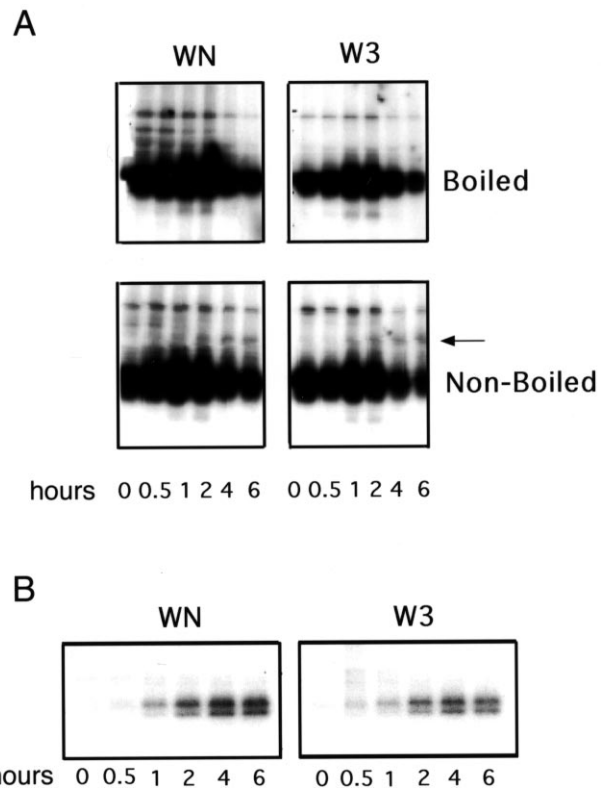

Fig. 3. The formation of SDS-resistant compact MHC-II dimers and mature MHC-II dimers is unaltered in HPS-2 patients. B-lymphoblasts from an HPS-2 patient (W3) and control B-lymphoblasts (WN) were labeled and processed as described in Fig. 2. In panel A, cell extracts were subjected to immunoprecipitation with an antibody to MHC-II α, and the washed immunoprecipitates were then eluted with SDS sample buffer, either by boiling (panel A, top) or incubating at room temperature (panel A, bottom). The arrow denotes the location of the SDS-resistant compact dimer. In panel B, immunoprecipitations were done with an antibody that recognizes mature, Ii-free MHC-II dimers.

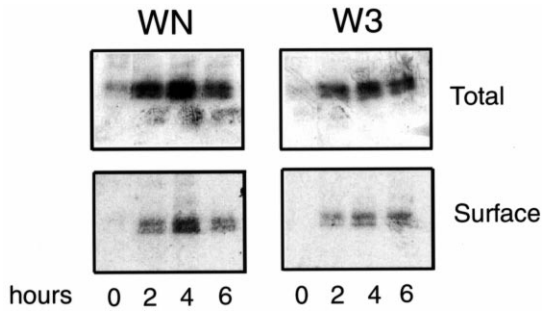

Fig. 4. The kinetics of cell surface expression of newly synthesized MHC-II dimers is similar in B-lymphoblasts of HPS-2 patients and normal controls. B-lymphoblasts derived from an HPS-2 patient and a normal individual were metabolically labeled and chased as described in Fig. 2. At the indicated times, the cells were surface biotinylated for 2 min at 4°C. Cell lysates were precleared twice and immunoprecipitated with the L243 antibody, which recognizes mature Ii-free MHC-II dimers. Ten percent of the immunoprecipitate representing the total mature MHC-II dimers was then eluted and analyzed by 4–20% SDS–PAGE (total; upper panel). The remaining 90% of the immunoprecipitate was reimmunoprecipitated with streptavidin beads, and the eluate was analyzed by 4–20% SDS–PAGE (surface; lower panel). Autoradiograms were visualized by fluorography.

form functions distinct from that of LYST, the protein that is defective in CHS.

AP-3 has been shown to be involved in the recognition of dileucine-based sorting signals [18]. However, a recent study has reported that the dileucine-based sorting signals of Ii, in particular, do not interact with the AP-3 complex in vitro [26]. Our findings are in line with this observation and suggest that a different AP complex mediates sorting of MHC-II to antigen-processing compartments. This role might be fulfilled by the related AP-1 and AP-2 complexes, which have been shown to interact with the cytosolic tails of Ii and the MHC Class II β chain [26–28]. Indeed, it has been demonstrated that overexpression of αβIi complexes induces recruitment of AP-1 to Golgi membranes [29].

HPS-2 patients have been reported to suffer from recurrent bacterial infections of the upper respiratory tract and inner ear [19]. The observations reported here suggest that these infections are unlikely to result from deficient antigen presentation. Another defect, such as the persistent neutropenia observed for these patients [19], may well be the cause for the increased susceptibility to bacterial infections.

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